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Puromycin aminonucleoside metabolism by glomeruli and glomerular epithelial cells in vitro

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Puromycin aminonucleoside metabolism by glomeruli and glomerular epithelial cells in vitro. Two puromycin aminonucleoside (PAN) excretion products were purified by HPLC from urine of PAN-treated rats and characterized by nuclear magnetic resonance as N^6-dimethyl-3′amino-3′deoxyadenosine (DA-Ado) and N^6-methyl-3′amino-3′deoxyadenosine (MA-Ado), respectively, the former corresponding to unmodified PAN. DA-Ado was not a substrate for adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) or xanthine oxidase (XO), while MA-Ado was consecutively converted into hypoxanthine by a mixture of ADA and PNP. A different rate of transformation of DA-Ado and MA-Ado into hypoxanthine by isolated glomeruli was observed and was higher for the monomethylated analogue by a factor of 3 (79% vs. 21%); this was ascribed to the rate-limiting level of a demethylase activity acting on DA-Ado. Furthermore, DA-Ado was not transformed by glomerular epithelial cells in culture, while a little amount of MA-Ado was converted into hypoxanthine after six hours of incubation. In spite of this different metabolic behavior, the same order of cytotoxicity on glomerular epithelial cells in culture was observed for MA-Ado, DA-Ado and commercial PAN. All these molecules induced a dose response inhibition of [^{3}H]thymidine incorporation into DNA after exposure for two hours and a marked alteration of cell viability which was not inhibited by free radical scavengers and deferoxamine. This study provides the first evidence for a glomerular metabolism of PAN and its urinary metabolite MA-Ado involving their transformation via the purine cycle enzymes. The rate of glomerular transformation of PAN in isolated glomeruli and the lack of its metabolism in cultured epithelial cells implies the presence of demethylase enzymes within the glomerulus and their absence in cultured epithelial cells. However, the similar cytotoxic effect of PAN and MA-Ado on cultured epithelial cells supports the concept that a mechanism which does not involve the purine cycle is responsible for PAN glomerular toxicity in vitro.

The administration of PAN to rats results in massive proteinuria due to well-defined glomerular lesions which are similar in many aspects to human minimal change nephropathy [1]. While the structural defect responsible for proteinuria has been identified in an alteration of the *de novo* proteoglycan synthesis by glomerular epithelial cells [2–4] accompanied by cell detachment [5] and/or in changes of the GBM polyanion composition [6], little is currently known on the mechanisms responsible for them. In recent years a role for toxic oxygen radicals in PAN nephrotoxicity has been tentatively hypothesized [7–10] on the basis of a protective effect on proteinuria of superoxide dismutase and of other scavengers such as DMTU [9], benzoate [9] and polyethylene glycol-conjugated catalase [10]. Other independent findings which reinforce the hypothesis of free radical implication in PAN nephrosis are that proteinuria is reduced by the iron chelator deferoxamine which inhibits the synthesis of $OH^\bullet$ via the Haber-Weiss reaction [9]. The same effect was also reported when xanthine oxidase was inhibited by allopurinol [8]. From all these data, a pathogenetic hypothesis has arisen where PAN is first converted to hypoxanthine within the kidney and that the successive conversion of hypoxanthine to uric acid via the xanthine oxidase system is responsible for $O_2^\bullet$ generation [8]. A number of crucial aspects regarding the metabolic pathway for $O_2^\bullet$ generation from PAN remain, however, largely hypothetical. First of all, the putative metabolic pathway for transformation of PAN into hypoxanthine within the glomerulus and its potential nephrotoxicity must be clarified, since the increased supply of hypoxanthine via direct infusion of the base does not induce proteinuria while being metabolized within the kidney [11]. In a second instance, the lack of knowledge about glomerular PAN metabolism contributes in making any consideration on drug renal toxicity unclear and any conclusion arbitrary. The central questions are whether PAN is metabolized by glomerular cells or not and whether PAN or any of the PAN metabolites produced in vivo are substrates for enzymes of the purine cycle (ADA, PNP and XO, the last one being responsible for the conversion of hypoxanthine to uric acid). All these crucial points are addressed in this paper.

Methods

Materials

PAN, adenosine deaminase (E.C. 3.5.4.4), purine nucleoside phosphorylase (E.C. 2.4.2.1), xanthine oxidase (E.C. 1.1.3.2.2), ethylendiaminetetraacetate (EDTA), trypsin inhibitor, dithiothreitol (DTT) were obtained from Sigma (St. Louis, Missouri, USA); reverse-phase chromatography columns RP18 and preparative C18 cartridges Sep-Pack were from Waters (Milford, Massachusetts, USA). Coomassie G-250 was from Fluka (Buchs, Switzerland); RPMI 1640 and 199 medium,

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penicillin, streptomycin, L-glutamine and Trypan Blue were from Flow Laboratories (Milan, Italy); bovine type I collagen, Vitrogen, was from Collagen Corporation (Palo Alto, California, USA); [^{3}H]thymidine (5 Ci/mmol) was from Amersham (Buckinghamshire, UK); bacterial collagenase was from Worthington (Freehold, New Jersey, USA).

Animal model

Sprague-Dawley/CD male rats (Charles River, Calco, Italy) weighting 200 to 250 g at the start of the experiment were used. PAN nephrosis was induced by a single injection of PAN (7.5 mg/100 g dissolved in 0.5 ml of normal saline) through the tail vein of non-anesthetized animals. Control rats were given normal saline. In all cases rats were fed a standard diet ad libitum containing 20% (wt/wt) casein and 2% (wt/wt) trace elements (non-tungsten) and providing 3,400 kcal/kg (Altromin-Rieper, Vandoies, Italy), and were allowed free access to water.

Determination and purification of PAN metabolites

Urine samples were deproteinized by precipitation with 12% trichloracetic acid (1:5 vol/vol) at 4°C for 15 minutes, followed by protein removal by centrifugation at 2,000 · *g* (15 min, 4°C) and neutralization by KOH 2 M. An HPLC system was used to separate and determine PAN metabolites, consisting in a reverse-phase column (C 18) working at room temperature and employing a three-step mobile phase: (1) constant 0.025 mol/liter KH_2PO_4 pH 5 with a flow rate of 1 ml/min for 15 minutes; (2) a linear gradient between phase 1 and 30% (vol/vol) methanol for 20 minutes with a 1 ml/min flow rate; and (3) constant 0.025 mol/liter KH_2PO_4 30% (vol/vol) methanol for 15 minutes with a 1 ml/min flow rate. A variable wavelength visible, U.V. detector was employed to analyze the spectral characteristics of eluted metabolites whose concentrations were calculated by comparison with a standard sample of PAN of known concentration. The same apparatus was employed for purification of every single metabolite, and each was further processed by preparative reverse-phase chromatography on a C 18 Sep-Pack column working at room temperature, and pretreated with 10 ml of 100% (vol/vol) methanol followed by 10 ml H_2O. After sample loading (4 ml), the separation was developed by 4 ml H_2O (to eliminate salts), and 2 ml 100% methanol in which phase every single metabolite was eluted. Purity was tested by HPLC as described above.

Determination of hypoxanthine, xanthine and uric acid

Hypoxanthine and xanthine were determined by reverse-phase HPLC under the same conditions used for PAN metabolites. Uric acid was determined by a previously-reported enzymatic assay [11].

NMR spectra

^{1}H-NMR spectra were obtained with a Bruker AM-270 spectrometer, controlled by an Aspect 3000 Computer. The 2D-COSY spectrum was acquired with a sweep width of 1800 Hz over 2K data points and resolution-enhancement was obtained with a $\pi/3$-shifted squared sine-bell in both dimensions. Chemical shifts were given in ppm from sodium 3-trimethylsilyl (2,2,3,3-^{2}H4) propionate (TSP). Assignments of proton spectra of the two urinary compounds were done by comparison with the spectrum of commercial PAN.

ADA, PNP and XO determination

ADA activity was assayed spectrophotometrically at 265 nm, using as substrate 0.1 mM adenosine in 0.1 M Tris-HCl buffer, pH 7.4. PNP activity was assayed spectrophometrically at 293 nm, in the presence of excess commercial xanthine oxidase, using as substrate 0.9 mM inosine in 0.1 M Tris-phosphate buffer, pH 7.4. XO activity was assayed spectrophotometrically at 293 nm, using as substrate 0.9 mM hypoxanthine in 0.1 M Tris-HCl buffer, pH 7.4. All assays were performed at 37°C in 1 ml reaction mixtures.

Tests with enzymes

Fractions purified by HPLC as described above, starting from the urine of rats treated with PAN, were challenged with some purine metabolizing enzymes: adenosine deaminase (ADA), nucleoside phosphorylase (PNP) and xanthine oxidase (XO). The assay was carried out with commercial enzymes in 0.15 M Tris-phosphate buffer pH 7.4 at 25°C. The reaction mixture contained, in a final volume of 1 ml, 50 nmol of the tested compound and 1 enzyme unit of each enzyme. Reactions were monitored both by recording spectra between 300 and 240 nm every minute over a 10 minute interval and by injection in the HPLC apparatus (under the conditions specified above) at the end of a one hour incubation period.

Isolation of glomeruli

Glomeruli were isolated from Sprague-Dawley/CD male rats by a differential sieving technique according to standard methods [12]. Sprague-Dawley rats weighing 150 to 180 g were anesthetized with Ketalar (30 mg/kg). After clamping the aorta, the renal veins were cut and kidneys were perfused with ice-cold 0.9% NaCl containing 1 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 5 μg/ml trypsin inhibitor and 2 U/ml Trasylol, and kidneys were removed after blanching. The dissected cortex was then minced with a razor blade in PBS containing the same protease inhibitors and gently squeezed through a stainless steel sieve (250 μm). The resulting suspension was filtered through a 150 μm sieve and was washed three times in HBSS. Centrifuged glomeruli were finally collected on the top of a 75 μm sieve. The retained glomeruli were rinsed in PBS and tested by microscopy: only preparations with > 95% glomeruli were used. The glomerular suspension was centrifuged at 150 · *g* for five minutes and resuspended in a small volume of 50 mM Tris-HCl buffer, pH 7.5, containing protease inhibitors. After disruption of cellular structures by sonication, protein content was determined using the Coomassie dye binding assay [13].

Cell culture

Glomerular epithelial cells were cultured starting from purified glomeruli obtained from Sprague-Dawley rats (150 to 180 g) and killed with a lethal dose of sodium pentobarbital. Glomeruli isolated by the sieving technique (see above) under sterile conditions were resuspended in 199 medium supplemented with 10% fetal calf serum (FCS), 2.2 g/liter sodium bicarbonate, 2 mmol/liter glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.18 U/ml insulin. They were then plated onto 25-sq cm Falcon tissue culture flasks, previously coated with a thin film

of collagen and maintained at 37°C in humidified 5% CO_2 atmosphere. Cultured cells were recognized as epithelial [14] from: (1) typical cobblestone-like morphology and polygonal shape; (2) early outgrowth; (3) absence of factor VIII as determined by double immunofluorescent technique; (4) positivity for cytokeratin. To avoid mesangial cell contamination, only cells obtained from the primary culture (1 to 2 weeks) were used. Mesangial cells were recognized from epithelial cells based on the following criteria: (1) morphology; (2) negative staining by indirect immunofluorescence for cytokeratin; (3) contractile response to angiotensin II. Cells deriving from the second or third subculture were used.

Incubations

Incubations of glomeruli homogenates (protein content: 2 mg/ml) and GEC (10^4 cells under a 5% CO_2 atmosphere) with commercial PAN and PAN metabolites were performed at 37°C in 0.05 M Tris-HCl buffer pH 7.5, containing 1 mM EDTA, 0.2 mM PMSF, 1 mM DTT and 5 μg/ml trypsin inhibitor. Freshly prepared glomeruli were dissolved in 0.05 M Tris-HCl buffer and sonicated at 0°C until complete tissue disruption. Equimolar (1 mmol/liter) concentrations of PAN or PAN metabolites (PAN-Sigma, DA-Ado and MA-Ado) were added to experimental tubes. The control tube received buffer in equal volume. A total of 0.3 mM NADPH was added in the test tubes. In a typical experiment, incubation was continued for two hours in a final volume of 500 μl and was then stopped by adding 500 μl of ice-cold 10% trichloracetic acid. After 20 minutes, samples were centrifuged for 20 minutes at 2,000 · g. Following neutralization with 2 M KOH, the supernatant was injected in the HPLC apparatus.

Cytotoxicity assays

After exposure to PAN or DA-Ado or MA-Ado, [^{3}H] thymidine incorporation by GEC was evaluated by incubating 10^4 cells with 5 μCi/ml [^{3}H]thymidine for 24 hours at 37°C in a 5% CO_2 atmosphere. Dose response curves for PAN toxicity were performed by incubating PAN in several concentrations from 0.1 to 2 mM. Free radical scavengers (Allopurinol 1 mM; Catalase 2,000 U/ml) were preincubated with cells for two hours to permit their free passage within the cell, and were removed prior to the addition of PAN. In the case of deferoxamine, cells were preincubated with iron chelator (1 to 5 mM) for four hours by the method according to Gannon et al [15]. On completion of the drug exposure, cells were washed twice and resuspended in fresh medium. After extensive washing in RPMI, cells were counted in a Packard β-scintillation counter after being transferred under vacuum to filter paper sheets, and were solubilized in filter count scintillation fluid. All determinations were performed in triplicate. For measurement of cell viability, the Trypan-blue dye exclusion test was employed [16]. Cultures were harvested in 0.05% trypsin - 0.02% EDTA and stained with 0.05% Trypan blue for five minutes. The percent of cells excluding the dye was then evaluated by direct counting in a hemocytometer.

Other methods

The Coomassie dye binding assay [13] was employed to evaluate proteinuria.

Statistical analysis

The one-way analysis of variance was used in all statistical tests. Results are given as mean ± SE.

Results

Purification and characterization of PAN metabolites from rat urine

Two chromatographic peaks, corresponding to PAN analogues, were isolated from urine of PAN-treated rats by reverse phase HPLC. The slower one had the same chromatographic and optical (absorbance maximum at 275 nm) properties of unmodified PAN, while the faster one eluted before PAN and presented an absorbance maximum at 266 nm. The two peaks were purified by preparative reverse-phase HPLC and were characterized by NMR. Spectral characteristics and structural assignment are reported in Figure 1 and in Table 1. By comparing the spectra from PAN, two main features were observed: (1) the intensity of the methyl group corresponded to six protons in PAN and in the slower peak, while to three protons in the other, indicating the presence of only one moiety; (2) the chemical shifts of protons H2 and H8 were slightly different between the two purified metabolites, the major one corresponding to PAN. The slower peak corresponded to the N^6-dimethyl-3′amino-3′deoxyadenosine and was therefore labeled as "DA-Ado". The faster peak, on the other hand, contained the monomethylated form of this molecule, namely N^6-methyl-3′amino-3′deoxyadenosine, and was labeled as "MA-Ado".

Urinary excretion of DA-Ado and MA-Ado

The rate of urinary excretion of both metabolites are reported in Figure 2 and in Table 2, respectively. As shown, DA-Ado and MA-Ado were rapidly (within 6 hr) excreted into urine, and no trace of either peak was found in urine after 24 hours. The total amount of PAN excreted into urine in the 24 hours following PAN treatment in the form of unmodified molecule (DA-Ado), and MA-Ado was the 50% of the injected compound. The main fraction (DA-Ado) roughly corresponded to the 70% of the total urinary excretion.

In vitro metabolism of PAN and PAN products by purine cycle enzymes

Activities of the purine cycle enzymes, ADA, PNP and XO, on commercial PAN and its urinary products DA-Ado and MA-Ado are shown in Table 3. While in this in vitro system commercial PAN and DA-Ado were not substrates for any of the three enzymes or for a mixture of them, MA-Ado was transformed by ADA in a product (presumably 3′-amino-3′deoxy-ribosylhypoxanthine) which in turn was transformed by PNP into hypoxanthine and eventually into urate by XO. This transformation by the in vitro system of MA-Ado into urate was complete within one hour of incubation at 25°C. Any other combination of enzyme activity not following the scheme ADA→PNP→XO did not produce any change in MA-Ado (Table 4).

Metabolism of PAN and PAN products by isolated glomeruli and glomerular epithelial cells in culture

We first investigated whether isolated glomeruli contained the enzymes of the purine cycle. Secondly we focused on the

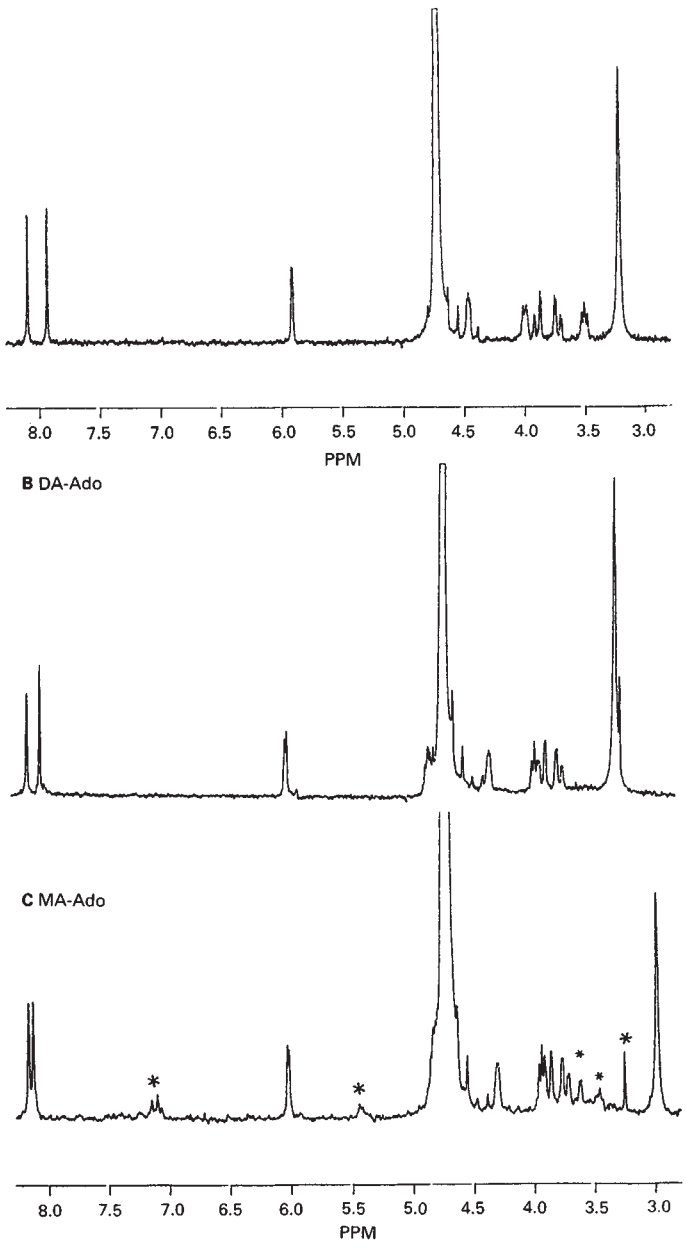

Fig. 1. *NMR spectra and structure assignment for PAN and the two urinary analogs.* The structural assignment of each peak are shown in Table 1. (*) Indicates the presence of impurities.

Table 1. Chemical shifts in ppm relative to TSP (sodium 3-trimethylsilyl (2,2,3,3-^{2}H4) propionate

	PAN	DA-Ado	MA-Ado
H-8	8.08	8.13	8.15
H-2	7.92	8.02	8.11
H-1	5.90	5.99	6.00
H-2	not observed	4.82	4.81
H-4	4.45	4.32	4.28
H-3	3.99	3.94	3.92
H-5	3.88	3.88	3.87
H-5′	3.71	3.73	3.72
Me	3.20	3.28	2.97

Me indicates the methyl group.

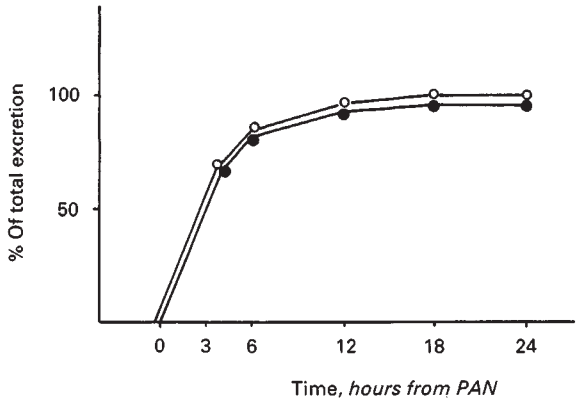

Fig. 2. *Urinary excretion of DA-Ado (○—○) and MA-Ado (●—●) following administration of PAN to rats.* Results are given as percent of the total urinary excretion of each metabolite.

Table 2. Urinary excretion of the two major analogs of PAN, DA-Ado and MA-Ado in the 24 hours which followed PAN injection in rats

Analog	*N*	mg/24 hr	% of injected PAN
MA-Ado	13	1.95 ± 0.22	10.6 ± 1.1
DA-Ado	13	6.71 ± 0.93	36.3 ± 4.7
Total		8.66 ± 1.09	46.9 ± 6.1

glomerular metabolism of PAN and related products. In glomeruli, all three enzymes which constituted the in vitro system acting on MA-Ado were present (Table 5). The specific activity of these enzymes followed the same pattern already described for renal homogenates [17, 18], and for other organs such as heart and liver where PNP level is 1.5 times the ADA levels and is higher compared to XO by a factor of 30. Due to this fact, MA-Ado was efficiently converted into hypoxanthine (79% decrement of MA-Ado and 79% increment of hypoxanthine) upon incubation for six hours (Table 4). This metabolic rate was consistently lower in the presence of cultured GEC where only 10% of MA-Ado was converted into hypoxanthine. Interestingly, DA-Ado was also metabolized by isolated glomeruli, about 20% of this compound being converted to hypoxanthine, while (as already shown above) no metabolism of Da-Ado was achieved with a mixture of PNP and ADA in vitro (Table 4). This suggests the presence of demethylase enzymes within the glomerulus which would convert PAN (or DA-Ado) into MA-Ado. As for MA-Ado, GEC are not the site for DA-Ado transformation in spite of the fact that a considerable amount (>50%) of both molecules entered the cells. Figure 3 shows the time-dependent transformation of PAN and DA-Ado into hypoxanthine with isolated glomeruli; more than four hours elapse before the appearance of PAN metabolites.

In vitro cytotoxicity

In vitro cytotoxicity of PAN and its analogues on cultured GEC was investigated by two tests: the former, based on

Table 3. Activities of the purine cycle enzymes, ADA, PNP and XO on PAN and on the related products, DA-Ado and MA-Ado, purified from urine of PAN-treated rats

Commercial enzyme	Activity relative to adenosine or inosine			
	PAN	DA-Ado	MA-Ado	$3'NH_2$-RHx
ADA	0	0	$3.6 \cdot 10^{-4}$	—
PNP	0	0	0	0.012
XO	0	0	0	0

$3'NH_2$-RHx ($3'NH_2$-ribosyl hypoxanthine) indicates the product of transformation of MA-Ado by ADA.

Table 4. In vitro metabolism of PAN and of its urinary products, DA-Ado and MA-Ado, purified from rats treated with PAN, as evaluated by HPLC analysis after incubation with the appropriate enzyme system

In vitro system	PAN		DA-Ado		MA-Ado	
	% Hypo	% PAN	% Hypo	% DA-Ado	% Hypo	% MA-Ado
ADA[a]	0	0	0	0	0	0
PNP[a]	0	0	0	0	0	0
ADA + PNP[a]	0	0	0	0	+100 ± 0.1	−100 ± 0.1
Isolated glomeruli[b] ($N = 4$)	+21 ± 3	−21 ± 3	+21 ± 3	−21 ± 3	+79 ± 0.4	−79 ± 0.4
Cultured podocytes[c] ($N = 4$)	0	0	0	0	0	0

[a] One hr incubation at 25°C in 0.1 M phosphate buffer pH 7.4
[b] Six hr incubation at 37°C in 0.1 M phosphate buffer pH 7.4 in the presence of 0.3 mM NADPH
[c] Six hr incubation with 10^4 cells

Table 5. Enzymatic activities of adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO) in isolated rat glomeruli

N	Enzymatic activity *EU* mg^{-1}
ADA *3*	0.093 ± 0.005
PNP *3*	0.13 ± 0.01
XO *3*	$0.5 \cdot 10^{-3}$ ± 0.01

Data are given as enzymatic units per milligram of protein.

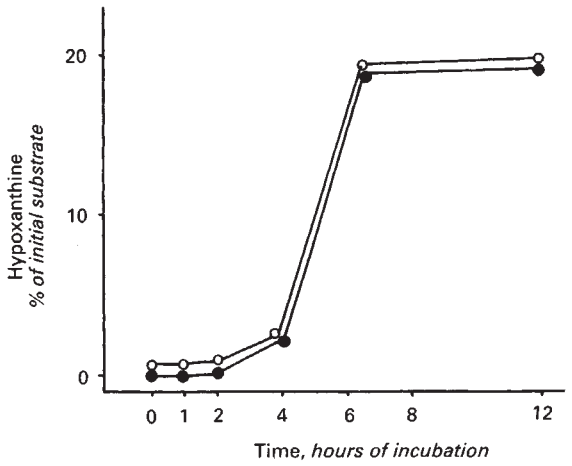

Fig. 3. *Rate of glomerular hypoxanthine production from PAN (●—●) and DA-Ado (○—○) as a function of incubation time.* Results are given as percent of the initial amount of substrate on a molar basis.

Table 6. In vitro cytotoxicity on GEC by PAN and its urinary products DA-Ado and MA-Ado

Treatment	[^{3}H]Thymidine incorporation *cpm/10^4 cells*	Cell viability *% of total cells*	
		1 day	5 days
None	10,400 ± 600 (100%)	97 ± 1%	76 ± 1%
1 mM PAN	1,040 ± 110 (10%)[a]	77 ± 9%[a]	34 ± 1%[a]
1 mM DA-Ado	1,476 ± 72 (14.2%)[a]	80 ± 4%[a]	40 ± 2%[a]
1 mM MA-Ado	1,248 ± 24 (12%)[a]	82 ± 2%[a]	36 ± 4%[a]
1 mM Hypoxanthine	10,000 ± 351 (100%)	96 ± 6%	79 ± 3%

Twenty-four-hour [^{3}H]thymidine incorporation was evaluated after incubating cells for 2 hours in the presence of each compound. Cell viability was evaluated by the Trypan-Blue dye exclusion test.
[a] $P < 0.001$ vs. control cells (no treatment)

24-hour [^{3}H]thymidine incorporation into DNA, was planned to assess the acute effects of aminonucleosides; the second test was aimed more directly at analyzing the long-term effect on cell viability. Both tests demonstrated a marked cytotoxic effect of all molecules which induced comparable inhibitory effects on [^{3}H]thymidine incorporation into GEC DNA and on cell viability (Table 6). The dose-response cytotoxicity curve of PAN and related metabolites on GEC is shown in Table 7. Here it appears that even the lower dose of PAN (0.1 mM) was able to inhibit DNA synthesis by 50%, as well as DA-Ado and MA-Ado. In no case did the use of allopurinol and catalase as free radical scavengers and of the iron chelator deferoxamine show any protective effect against PAN induced cytotoxicity (Table 7).

Discussion

Recent hypotheses on a role for free oxygen radicals in PAN-induced proteinuria were based on a protective effect of scavengers such as DMTU, benzoate, SOD and PEG-conjugated catalase [7–10]. Moreover, the protective effect of the iron chelator deferoxamine [9] suggested that iron is critical in the generation of free radicals from PAN, lending credence to the idea that $OH^\bullet$ arises from $O_2^\bullet$ via the Haber-Weiss reaction [19]. The central point regarding the mechanism(s) of glomerular toxicity by PAN, which is so far unresolved, is the question on the metabolic pathway generating $O_2^\bullet$ from PAN which should be reasonably active within the glomerulus due to the very short lifetime (in the order of milliseconds) of the implicated radicals. The most favored hypothesis, proposed by Diamond, Bonventre and Karnovski [8] states that hypoxanthine, a minor putative metabolite of PAN [21], represents the source of $O_2^\bullet$ via the xanthine oxidase system which transforms hypoxanthine into xanthine and uric acid. Preliminary studies on free radical generation from PAN by GEC in vitro have also provided the theoretical support for this possibility [20].

Too little is currently known about the general metabolism of PAN in rats, and more specifically about the glomerulus as the site of PAN transformation, to tempt any alternative interpretation. This study was therefore designed to define the metabolic pathways within the glomerulus for PAN and its major metabolic products. The data reported confirm previous obser-

Table 7. Dose-response of in vitro cytotoxicity on GEC by PAN metabolites

Treatment	[^{3}H] Thymidine incorporation *cpm/10^4/cells*
None (5)	9,851 ± 632 (100%)
0.1 mM PAN (5)	5,221 ± 131 (53%)
0.5 mM PAN (4)	3,940 ± 262 (40%)
1 mM PAN (4)	988 ± 56 (10%)
2 mM PAN (4)	838 ± 21 (8.5%)
0.5 mM PAN + Def (4)	4,137 ± 231 (42%)
0.5 mM PAN + Allo (4)	3,546 ± 128 (36%)
0.5 mM PAN + Cat (4)	3,841 ± 323 (39%)
1 mM PAN + Def (5)	1,182 ± 63 (12%)
1 mM PAN + Allo (5)	1,083 ± 89 (11%)
1 mM PAN + Cat (4)	830 ± 10 (9%)
0.1 mM DA-ADO (2)	5,810 ± 60 (59%)
1 mM DA-ADO (2)	1,398 ± 220 (14.2%)
0.1 mM MA-ADO (2)	5,122 ± 280 (52%)
1 mM MA-ADO (2)	1,180 ± 100 (12%)

Several free radical scavengers were employed for tentatively block the cytotoxicity of PAN. Abbreviations are: Def, deferoxamine; Allo, allopurinol; Cat, catalase.

vations demonstrating that no more than 50% of injected PAN is excreted into urine [21, 22], and give for the first time spectroscopic evidence for the in vivo formation of a urinary metabolite which corresponds to the monomethyl derivative. Both unmodified PAN and its monomethyl derivate are extensively cytotoxic on cultured GEC in vitro when evaluated by [^{3}H]thymidine incorporation into DNA and by the Trypan blue exclusion test, with an order of toxicity greater for the monomethylated analogue compared to the dimethylated form. Although these measures of cytotoxicity may not reflect the precise mechanisms that are responsible for proteinuria, they have been widely employed in the past and represent reliable markers of toxicity in vitro [16]. From other studies, glomerular epithelial cells have been shown to be the target of PAN cytotoxicity in vitro where it produces focal detachment of cells, alteration in polyanions synthesis and changes in the turnover of glucosamine [23]. All these changes are considered to be the main pathogenetic factor in determining proteinuria in this model of nephropathy [24].

The primary finding reported in this study is that both PAN derivatives can be partially metabolized within the glomerulus in relation to the presence of one or two methyl groups at N^6. A scheme of the pathway for transformation of both DA-Ado and its monomethylated analogue is shown in Figure 4, which sums up the in vitro data obtained from an artificial metabolic pathway and from isolated glomeruli. In the presence of glomerular homogenates, both metabolites are partially converted to hypoxanthine. Quantitative evaluation of the data, however, shows that in glomeruli the rate-limiting factor for the metabolization of the dimethyl compound is the first step, relative to formation of the monomethylated analogue by a demethylase activity. Indeed, due to the high level of both PNP and ADA specific activities, conversion of MA-Ado to hypoxanthine is not expected to be a rate-limiting step in the overall scheme. These data strongly support the concept that some PAN is transformed by glomerular homogenates into the precursors of uric acid, but the main quota of this molecule is not transformed by renal tissue. The lack of conversion of PAN into hypoxanthine by GEC suggests the absence of demethylases in these cells, which could also possibly be due to an in vitro artifact. In any case, PAN may be converted in vivo by endothelial or mesangial cells which may contain demethylase. All these points raise important questions about the mechanism for PAN toxicity in vivo. If in fact we stress the concept that PAN is actively cytotoxic on the kidney through its monomethylated analogue following transformation to substrates for the XO system (hypoxanthine and xanthine) to be converted into uric acid, the block of the rate limiting enzyme for this metabolic pathway, namely the demethylase, should confer protection against proteinuria in this model. Data in the literature are unfortunately quite confusing and do not permit a definite conclusion. The three following points speak against any implication of the glomerular metabolism of PAN via the purine cycle enzymes as a source of nephrotoxic compound: (1) in vivo inhibition of microsomal N-demethylase (cytochrome P-450) by α-naphthylisothiocyanate or SKF-525A did not reduce proteinuria in PAN treated rats [25]; (2) the mechanism of action of PAN in vivo is very fast [1] (a matter of a few minutes) while in vitro conversion of PAN into hypoxanthine within the glomerulus employs several hours (more than 2 hours); (3) hypoxanthine is not directly cytotoxic on cultured podocytes in vitro, even when given in very high doses to overload the intracellular compartment (data reported here). Furthermore, studies on the mechanism of PAN action in vivo and in vitro have revealed that some structural analogues of PAN containing a 3′-hydroxyl group, such as N^6-methyl- and N^6-dimethyl-adenosine are active blockers of PAN nephrotoxicity through competition at the target of aminonucleoside action and/or of its cellular uptake [25, 26]. The former molecule is a substrate for ADA and is eventually transformed to hypoxanthine in vitro at rate at least similar to that of MA-Ado, thus excluding any direct implication of this pathway in nephrotoxicity. This point needs, however, further study which should point out the effectiveness of demethylase inhibitors at the cellular level as blockers of PAN toxicity in vitro. In spite of the uncertainties about the in vivo mechanism of PAN toxicity, some conclusions on the in vitro mechanism of cytotoxicity may be reached. The occurrence of PAN toxicity on GEC without conversion into hypoxanthine implies that alternative mechanisms for the inhibition of DNA synthesis and successive cellular death are active in this condition. The lack of a protective effect on PAN cytotoxicity by free radical scavengers or by a potent inhibitor of $OH^\bullet$ formation, such as the iron chelator deferoxamine, also supports this view. Determining the mechanism responsible for PAN cytotoxicity that does not involve the purine cycle metabolism was beyond the scope of this work, however, on a theoretical ground we hypothesize that it may be ascribed to a general effect of PAN analogs on protein synthesis [27]. PAN is, in fact, able to interrupt the peptide chain elongation during protein synthesis in ribosomes, by virtue of the capacity to react with peptidyl-RNA, a reaction which yields peptidyl-PAN [27].

In summary, the data reported here provide the first evidence for a glomerular metabolism of PAN and its urinary products, DA-Ado and MA-Ado, involving their transformation via the purine cycle enzymes. The slow rate of transformation of DA-Ado by isolated glomeruli and the lack of its metabolism by

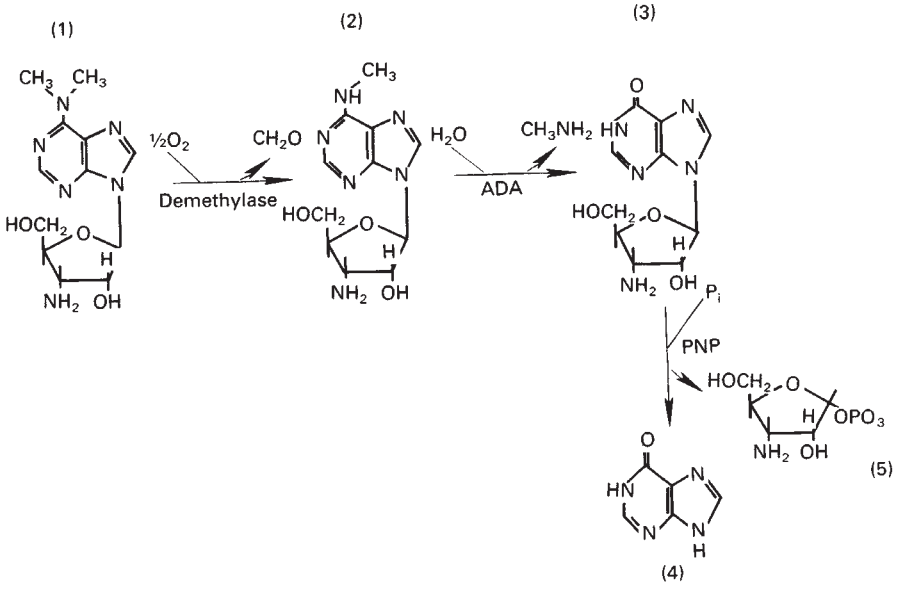

Fig. 4. *Putative pathway for the glomerular metabolization of the PAN analogues DA-Ado and MA-Ado, purified from urine of PAN-treated rats.*

cultured GEC implies the presence of demethylase enzymes within the glomerulus and their absence in cultured GEC. The similar cytotoxic effect of commercial PAN, DA-Ado and MA-Ado on these cells supports the concept that a different mechanism, not involving transformation of these compounds into hypoxanthine via the purine cycle enzymes, is responsible for its glomerular toxicity in vitro.

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